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Infection Chains and Evolution Rates of Hepatitis B Virus in Cardiac Transplant Recipients Infected Nosocomially

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Following an outbreak of hepatitis B virus (HBV) infection amongst immunosuppressed transplant recipients, the complete sequences of the HBV-DNA isolated from nine of the affected patients were determined. The DNA sequences were found to differ from each other by a maximum of three nucleotides and belonged to the same serotype (ayw3). By contrast, the sequences differed by 18 nucleotides from the most similar HBV-DNA sequence published, indicating a common source of infection. The infection chains that have been constructed according to the base differences between the DNAs agreed well with those previously established on the basis of epidemiological data [Drescher et al. (1994) Journal of Hospital Infection 26:81–92]. At least two HBV populations, differing by one or two nucleotides, were detected in four patients, and coexisted for differing periods of time. Mutations of the core and X-peptide were not found. The data were used to calculate evolution rates of HBV DNA, both for HBV persisting within a patient and for infection chains. The rates obtained were of the same order as described previously for immunocompetent patients, indicating that the immunosuppressive medication did not influence the evolution rate. However, the evolution rate was found to decrease with increasing evolution time. ***J. Med. Virol. 58:1–10, 1999.*** © 1999 Wiley-Liss, Inc.

KEY WORDS: HBV; DNA sequencing; "a" epitope; antigens; enhancer

INTRODUCTION

Mutants of hepatitis B virus (HBV) have been described to be associated with vaccine escape, loss of HBeAg, chronic hepatitis [Blum, 1995], diagnostic escape [Hou et al., 1995], therapy escape [Okamoto et al., 1992; Bartholomew et al., 1997], and perhaps the emergence of fulminant hepatitis [Kaneko et al., 1995; Sterneck et al., 1996, 1997]. Specific mutations have been used as markers for identifying chains of HBV transmission [Lin et al., 1991; Uy et al., 1992; Roll et al., 1995; von Weizsäcker et al., 1995; Zuckerman et al., 1995; Harpaz et al., 1996; Santantonio et al., 1997; The Incident Investigation Team et al., 1997].

The evolution of viable virus mutants is thought to result from random errors of the polymerase and their subsequent selection in the host. The evolution of the HBV has resulted in the appearance of different genotypes (A through F), serotypes, and strains, distributed inhomogeneously throughout the world [Couroucé-Pauty et al., 1983; Okamoto et al., 1988; Norder et al., 1992, 1993; Bowyer et al., 1997]. Furthermore, sequence differences have also been described for HBV isolated from serum, peripheral blood mononuclear cells, and liver tissue of the same immunosuppressed patient [Brind et al., 1997].

The frequency of genomic mutations can be used to calculate the evolution rate of viruses. Hepadnaviridae show higher evolution rates than other DNA viridae but lower rates than RNA viridae [Orito et al., 1989]. Evolution rates of HBV have been calculated to be in the range of $1–5 \times 10^{-5}$ and $5–8 \times 10^{-5}$ per site per year in immunocompetent patients [Orito et al., 1989; Lin et al., 1991], $6–18 \times 10^{-3}$ in liver transplant recipients with fulminant reinfection [Sterneck et al., 1997], and $0.3–1.3 \times 10^{-3}$ for the HBV core gene in a case of perinatally acquired chronic HBV infection [Bozkaya et al., 1997]. For woodchuck hepatitis virus, a rate of $\leq 2 \times 10^{-4}$ per site per year has been found [Girones and Miller, 1989]. Nucleotide substitutions in HBV-DNA often influence two peptides due to the overlap within half of the HBV genome [Blum, 1993]. Extensive deletions in the core and in the polymerase/surface gene (up to 120 and 1,210 nucleotides, respectively) have been found in the genomes of HBV populations isolated from kidney transplant patients with end-stage liver cirrhosis [Günther et al., 1995].

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TABLE I. Description of Patients and HBV-DNA Samples Investigated

Patient no.	Blood sample		Date of first detected HBsAg positivity	Calculated date of onset of HBsAg positivity[a]
	Drawn	HBV-DNA designated		
1	Oct. 6, 1986	1	Oct. 6, 1986	June 9, 1986
2	Feb. 19, 1988	2.1	Feb. 19, 1988	Aug. 2, 1987
	Feb. 16, 1994	2.2		
3	Nov. 4, 1988	3.1	Nov. 4, 1988	July 3, 1988
	Dec. 6, 1991	3.2		
4	Apr. 21, 1989	4.1	Feb. 24, 1988	Sept. 6, 1987
	Feb. 7, 1994	4.2		
5	Apr. 25, 1991	5	Nov. 13, 1990	Aug. 21, 1990
6	Apr. 29, 1991	6.1	Jan. 25, 1991	Dec. 7, 1990
	Aug. 24, 1995	6.2		
7	July 16, 1991	7	Nov. 21, 1990	Aug. 26, 1990
8	Dec. 10, 1991	8	Nov. 22, 1990	Oct. 27, 1990
9	May 20, 1992	9	Sept. 11, 1989	June 17, 1989

HBV, hepatitis B virus.
[a]The time interval between the date of last recorded HBsAg negativity and the date of first recorded HBsAg positivity was divided by two and the respective date considered as the date of onset of HBsAg positivity.

Between March 1986 and September 1990, 67 of 243 cardiac transplant recipients in outpatient care at the Department of Thoracic and Cardiovascular Surgery, the Medical School of Hannover, Germany, developed hepatitis B virus surface antigen (HBsAg) positivity after operation. The HBsAg of 63 patients was typed as ay, suggesting a common source of infection. The infections originated from an HBsAg carrier between these patients and were transmitted during endomyocardial biopsy, if performed on the same day and in the same room after biopsy of an HBsAg-positive patient. The mode of infection was droplet contamination of instruments and/or medication vials used for subsequent patients. A description of the analysis of this outbreak has been presented previously [Drescher et al., 1994].

The HBV-DNA of nine patients selected from this outbreak was sequenced completely to test whether the results could provide helpful information on the clonality of the HBV involved and on the infection chains, as described by Zuckerman et al. [1995] and Osterhaus et al. [1998]. In addition, these data were used to determine the evolution rate of HBV in immunosuppressed patients, both within a given patient and in infection chains comprising different patients.

MATERIALS AND METHODS

Patients

Nine cardiac transplant recipients (designated 1 through 9) with postoperative HBV infection were investigated. The dates on which the blood samples for DNA isolation were taken are given in Table I. The DNAs isolated were designated 1 through 9. If more than one blood sample of a patient was examined, the respective DNAs were earmarked by adding the symbol 1 or 2, respectively, and if more than one DNA population in the same sample of a patient was detected, the DNAs were described by adding the letters A and B. At the time of sampling, patients were HBsAg and HBeAg positive and five patients also showed Hbc IgM positivity (patients 1, 2, 3, 8, and 9). The sera contained about 10^8 HBV genome equivalents per milliliter.

All patients received standard immunosuppressive medication after transplantation, consisting of a combination of cyclosporine A (serum concentrations ranging from 100 to 300 ng/ml), azathioprine (1–2.5 mg/kg body weight per day), and prednisolone (0.1 mg/kg body weight per day). No patient developed clinically apparent hepatitis. Further data of these patients have been described previously [Drescher et al., 1994]; patient 1 was found to be the source of infection.

Isolation and Amplification of HBV-DNA

One hundred microliters of a mixture containing 89 μl serum, 60 mM Tris-HCl pH 8, 0.3% sodium dodecyl sulfate (SDS), 5 mM ethylenediamine tetraacetic acid (EDTA), 10 μg t-RNA as carrier, and 20 μg proteinase K (Boehringer, Mannheim, Germany) was incubated at increasing temperatures (37–56°C) for at least 4 hr or at 37°C overnight and extracted with phenol at 56°C and subsequently with chloroform. The DNA was precipitated with LiCl and ethanol at –20°C overnight. Pellets were rinsed with 70% ethanol, dried in a SpeedVac SC110A (Savant Instruments Inc., Farmingdale, NY), and dissolved in 10 μl TE (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA).

About 10^5 genome equivalents of HBV-DNA were amplified by polymerase chain reaction (PCR) in 50 μl reaction volumes containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 4 mM $MgCl_2$, dNTPs each 0.2 mM, two primers each 1 μM, and 7.5 U AmpliTaq DNA polymerase, Stoffel fragment (Perkin Elmer Roche Molecular Systems, Branchburg, NJ), using 0.2-ml PCR tubes and the "Programmable Thermal Controller PTC-100" with hot bonnet (MJ Research, Watertown, MA). The "hot start" PCR technique was used. The DNA in 45 μl reaction buffer was denatured for 5 min at 95°C and

held subsequently at 80°C, the polymerase and dNTPs dissolved in 5 μl buffer were added, and the final mixture was heated for 30 sec at 94°C. The first annealing and elongation steps were extended: for 3 min at 65°C and for 5 min at 72°C, respectively. The profile of the next 38 thermal cycles was 93°C for 60 sec, 66°C for 90 sec, and 72°C for 240 sec with an increment of 4 sec per cycle. The amplification was completed by heating at 72°C for 10 min.

The primer pairs used for amplifications were selected so that the amplified DNA region did not involve both double repeats. Poor PCR yields were thereby avoided. Amplificates with a grade of purity sufficient for cycle sequencing were obtained after optimisation of the PCR parameters, especially of the annealing temperature and elongation time.

Purification and Sequencing of the Amplificates

Amplified DNA was purified from low molecular compounds such as excess primers and smaller unspecific amplificates using Microcon 100 (Amicon, Beverly, MA). The amount of amplified DNA was estimated by comparing its band in the ethidium-bromide-stained agarose gel with bands of the DNA molecular weight markers III and/or VI (Boehringer Mannheim, Mannheim, Germany).

About 25 fmol purified DNA were cycle-sequenced, using 1.5 pmol 5′- biotinylated primer and the "fmol DNA Sequencing System" (Promega, Madison, WI). After a 2-min 95°C hold, 30 cycles were performed at 95°C for 30 sec, 67°C for 30 sec, and 70°C for 1 min. Routinely, about 500–600 bp were read in one run. The frequency of stops in the sequence ladder could be reduced by optimising the annealing temperature in both the amplification and the sequencing procedure and by using primers with binding sites located far from those of the primers used for amplification. Small amounts of short DNA fragment generated during the PCR process seemed to be responsible for the stops.

The amplificates were denoted according to the primers used: the amplificates J through A were sequenced with primers K, M, and N; amplificates M through E with primers A, B, and C; amplificates G through N with primers I and L; amplificates B through H with primers D and E; and amplificates F through L with primer G.

Primers

Nucleotide positions (in parentheses) were numbered according to the complete ayw HBV sequence of the HBV-DNA HBJAYW obtained from the EMBL database:

A* (399–377) GATAAAACGCCGCAGACACATCC,
B* (672–694) GCTCAGTTTACTAGTGCCATTTG,
C* (732–711) AAGCCAAACAGTGGGGGAAAGC,
D* (1,177–1,199) GCCAAGTGTTTGCTGAYGCAACC,
E* (1,284–1,264) GGAGTTCCGCAGTATGGATCG,
F (1,648–1,671) GGTCTTACATAAGAGGACTCTTGG,
G* (1,692–1,711) CCGACCTTGAGGCMTACTTC,
H* (1,759–1,736) CCTAATCTCCTCCCCCARCTCCTC,
I* (1,862–1,885) CTGTTCAAGCCTCCAAGATGTGCC,
J (2,413–2,433) CCGCGTCGCAGAAGATCTCAA,
K* (2,419–2,439) CGCAGAAGATCTCAATCTCGG,
L* (2,487–2,463) GTAAAGTTYCCSACCTTATGAGTCC,
M* (2,816–2,837) GGGTCACCATATTCTTGGGAAC,
N* (3,060–3,041) GAGCCTGMGGGCTCCACCCC.

Asterisks denote biotinylated primers used for sequencing. Five primers (D, G, H, L, N) contained degenerate bases that were homologous to the four known HBV serotypes as follows: adr in HBVADR [EMBL database V 00867; Ono et al., 1983], adw in HBVADW [EMBL database V 00866; Ono et al., 1983], ayr in HEHBVAYR [EMBL database X 04615; Okamoto et al., 1986], and ayw in HBJAYW [EMBL database J 02203; Galibert et al., 1979].

Identification of Different DNA Populations in a Sample

The presence of different DNA populations in a sample, detected by the appearance of double bands in the sequence ladder, was confirmed by sequencing the complementary DNA strand, or the use of a second amplificate and/or of another serum of the patient. The relative concentration of the DNA populations was estimated by comparing the respective intensities of the double bands.

Comparative Analysis of the Sequences

Calculation of the phylogenetic tree and the multiple alignments was performed using the program "Multalin, Version 3.0" [Corpet, 1989]. Sequence alignments were made using the following complete HBV ayw genomes: HBVAYWMCG, HBVAYWC, HBJAYW, HBVORFS, XXHEPAV, HPBHBVAA, HBVAYWCI, HPBMUT, HBVAYWE, HBVDNA from the EMBL database, the nine sequences "1" through "9" [Sterneck et al., 1996], the three sequences "1" through "3" [Sterneck et al., 1997], and the sequences BIE, LAU, and NAT [Kremsdorf et al., 1993].

Reconstruction of Infection Chains

The infection chains were reconstructed by using the following steps:

1. As a first step, the patients were arranged in order according to the probable dates of onset of HBsAg positivity and it was assumed that each of the HBsAg-positive patients could have infected any patient in whom HBsAg positivity developed later. All epidemiologically possible infection chains were thereby defined.
2. As a second step, the number of base differences between DNA samples was determined. If two DNA populations were present in the sample of a patient, the mean value was taken.

TABLE II. HBV-DNA Sequence Differences Between HBJAYW (First Letter) and the DNA[b] of Patient 1 (Second Letter), and Between the Amino Acids Deduced From Both Sequences

Nucleotides		Amino acids					
Position	Exchange	Peptide[a]	Position	Exchange	Peptide[a]	Position	Exchange
15	C→T	P	297	P→S	S2	8	F
82	A→G	P	319	N→S	S2	31	T→A
98	T→C	P	324	S	S2	36	L→P
178	T→C	P	351	I→T	S	8	F→L
348	T→C	P	408	L	S	64	S
465	G→A	P	447	V→I	S	103	M→I
501	A→C	P	459	N→H	S	115	T
522	G→A	P	466	D→N	S	122	R
584	C→T	P	486	F	S	143	S→L
872	A→G	P	582	L			
897	T→A	P	591	C→S			
1055	G→A	P	643	L			
1061	T→C	P	645	P			
1352	A→C	P	742	L			
1372	T→A	P	749	F→Y			
1511	C→T	P	795	F	X	46	P→S
1635	A→G				X	87	Q→R
1639	A→G				X	88	I→M
1678	A→T				X	101	S
1679	G→A				X	102	A→T
2142	G→A				C	80	A
2145	T→C				C	81	S
2244	A→C				C	114	T
2337	G→A	P	10	R→K	C	145	E
2491	G→T	P	61	G			
2605	C→T	P	99	L			
2647	C→T	P	113	A			
2848	G→T	P	180	Q→H			
3117	T→A	P	270	V→D	S1	90	S→T

[a]P, polymerase; S1, preS1; S2, preS2; S, surface; C, core; X, X-protein.
[b]accession no. AJ131956, EMBL Database.

The epidemiologically possible chains with the smallest number of base differences were considered to represent the probable infection chains.

Calculation of the HBV DNA Evolution Rates

The evolution rate (E) of HBV-DNA was calculated as follows:

$$E = N/(3{,}182 \times t)$$

where: N = number of nucleotide exchanges found when comparing the HBV-DNAs isolated from samples drawn at times 1 and 2; t = difference (years) in time between drawing samples 1 and 2; and 3,182 = number of bases of the HBV-DNA.

If the DNA samples compared were derived from different patients, the resulting evolution rate was denoted E_e and when using DNAs derived from the same patient the rate was designated E_i.

Statistical Methods

Mean values were tested for significant differences by means of the Mann–Whitney test. The influence on the evolution rates of evolution time and of the passing of virus in one or between different patients was determined by the general linear models (GLM)-analysis of variance (ANOVA). All tests were done by the Number Crunching Statistical System (NCSS). The significance level was .05, unless otherwise stated.

RESULTS

Subtyping of the HBV-DNAs Isolated From the Patients Studied

The base sequences of the HBV-DNAs isolated from the patients studied were compared with DNA sequences (EMBL database) characterising the four serotypes adr (HBVADR), adw (HBVADW), ayr (HEHBVAYR), and ayw (HBJAYW). HBJAYW showed the highest similarity of base sequence and the same number of base pairs (3,182), indicating that the isolated HBV-DNAs belonged to the genotype D [Okamoto et al., 1988]. Furthermore, they contained the 11 amino acids of the S antigen characteristic of the serotype ayw [Carman et al., 1990], and the presence of methionine-125 and threonine-127 indicated that they belonged to the serotype ayw3 [Magnius and Norder, 1995].

Comparison of the HBV-DNA Isolated From Patient 1 With HBJAYW

The HBV outbreak studied originated from patient no. 1. Therefore, DNA no. 1 represented the HBV transmitted to the other patients (= index virus). The

TABLE III. Nucleotide and Amino Acid Differences Between the Samples Tested

		Sample No.																
				2.2		3.1			4.1		4.2			6.1		6.2		
Position no.	Peptide	1 = 7 = 9	2.1	A	B^{+a}	A	B^{+}	3.2	A^{+}	B	A^{+}	B	5	A	B	A	B	8
Nucleotide # 57		C	*[b]	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*
Amino acid # 311	P	P	*	*	*	*	*	*	*	*	*	*	S	*	*	*	*	*
# 27	PreS2	F	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Nucleotide # 234		C	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*
Amino acid # 370	P	H	*	*	Q	*	*	*	*	*	*	*	*	*	*	*	*	*
# 25	S	L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Nucleotide # 465		A	*	*	*	*	G	*	*	*	*	*	G	*	*	*	*	*
Amino Acid # 447	P	I	*	*	*	*	V	*	*	*	*	*	V	*	*	*	*	*
# 103	S	I	*	*	*	*	M	*	*	*	*	*	M	*	*	*	*	*
Nucleotide # 584		T	*	*	*	*	*	*	C	*	C	*	*	*	*	*	*	*
Amino acid # 486	P	F	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
# 143	S	L	*	*	*	*	*	*	S	*	S	*	*	*	*	*	*	*
Nucleotide # 872		G	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*
Amino acid # 582	P	L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Nucleotide # 1130		A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C
Amino acid # 668	P	K	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	N
Nucleotide # 1163		A	C	*	*	*	*	*	*	*	*	*	*	C	C	*	*	*
Amino acid # 679	P	Q	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Nucleotide # 2786		A	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*
Amino acid # 160	P	R	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Nucleotide # 2942		T	*	*	*	*	*	*	*	*	*	*	*	C	*	C	*	*
Amino acid # 212	P	L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
# 31	PreS1	D	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

[a]+, major population.
[b]*, same base/amino acid as described for sample 1.

nucleotide and amino acid differences found when comparing DNA no. 1 and HBJAYW are summarised in Table II.

Note that 29 nucleotide exchanges were found, 4 of them yielding amino acid exchanges in two HBV peptides, 13 in only one peptide, and 12 silent exchanges. The X-peptide showed four (2.6%) amino acid substitutions, three of them in the nonoverlapping region. No amino acid exchanges were found in the longer core protein. The polymerase had 11 (1.3%) amino acid substitutions, 8 of which were in overlapping regions, and the large surface protein had 6 amino acid substitutions (1.5%), 3 of which were located at the small surface antigen, 1 in the preS1 region, and 2 in the preS2 region. In the region identified as the immunodominant epitope "a" (amino acids nos. 124–147), the serine in HBJAYW at position 143 was substituted by a leucine. The substitution 103 (M→I) in the S-antigen was located at a T-cell epitope [Rehermann et al., 1995], 90 (S→T) in the preS1 region, and 31 (T→A) and 36 (L→P) were located in a transcriptional activator domain [Hildt et al., 1996]. The 11 amino acid substitutions found in the polymerase were distributed over its four parts: 10 (R→K) near the *N*-terminus of the terminal peptide; 180 (Q→H), 270 (V→D), 297 (P→S), and 319 (N→S) at the tether; 351 (I→T), 447 (V→I), 459 (N→H), 466 (D→ N), and 591 (C→S) at the reverse transcriptase/DNA polymerase, upstream and downstream of motifs essential for its function [Radziwill et al., 1990]; and 749 (F→Y) at the RNase H. One amino acid substitution 46 (P→S) detected in the X-peptide was located in its *N*-terminal third, which is known to repress HBx transactivation [Murakami et al., 1994]. The other 3 amino acid exchanges [87 (Q→ R), 88 (I→M), and 102 (A→T)] were located in a region found to be essential for transactivation of virus and host genes [Murakami et al., 1994; Lee et al., 1995] and for binding of the TATA-binding protein [Qadri et al., 1995]. The 88-methionine resulted in a translation initiation in-frame codon producing an additional amino-terminally truncated X peptide [Kwee et al., 1992]. The exchange 102 (A→T) was located at the binding site of XAP3 protein [Cong et al., 1997].

Seven substitutions were found to belong to regulatory DNA regions: nucleotides at positions 1,055 (G→A) and 1,061 (T→C) were located in the region of the enhancer I. The exchanged nucleotides 1,635 (A→G), 1,639 (A→G), 1,678 (A→T), and 1,679 (G→A) were located within part A of the enhancer II, upstream and downstream of the binding site of protein A [Wu et al., 1992], and 1,678 and 1,679 within the γ-box and at the 5′ end (1,639) of the core upstream regulatory sequence (CURS) [Yuh et al., 1992]. The substitution at position 3,117 (T→A) was located in the element E of the major surface antigen promoter [Raney et al., 1992].

Comparison of the HBV-DNA Isolated From the Patients Studied

The differences between the base sequences and the deduced amino acids of the HBV-DNAs isolated from our patients are given in Table III. Two different HBV-DNA populations, denoted by the affixes A and B, were found to coexist in four patients (nos. 2, 3, 4, and 6). They coexisted in patients 4 and 6 in the same relative proportions for at least 5 and 4 years, respectively. The

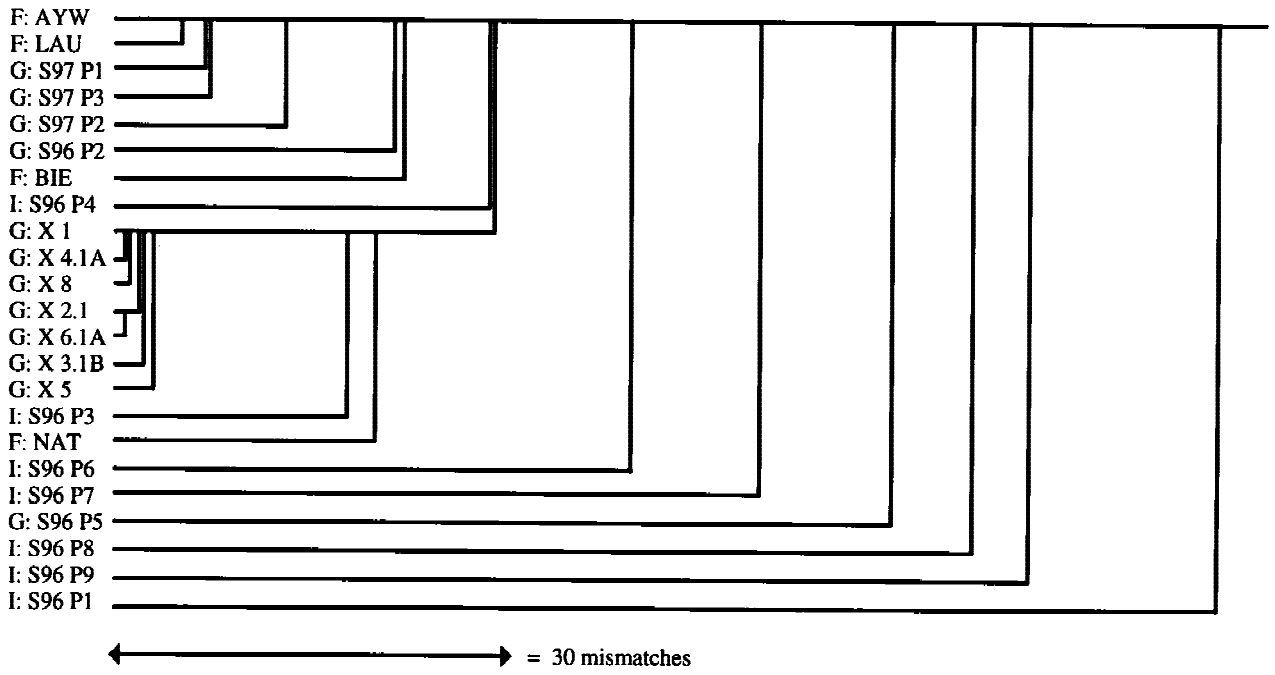

Fig. 1. Phylogenetic tree of European hepatitis B virus (HBV) isolates, serotype ayw. The lengths of the horizontal lines corresponded to the number of mismatches. The first letter indicates the country in which the HBV was isolated: F, France; G, Germany; I, Italy. AYW corresponds to HBJAYW [EMBL database; Galibert et al., 1979]. LAU, BIE, NAT were described by Kremsdorf et al. [1993]. X designates HBV populations (A and B) isolated from our HTX patients, 1 also stands for 3.1B, 4.1B, 7, and 9, and 2.1 for 6.1B. The samples earmarked S96 and S97 were described by Sterneck et al. [1996, 1997].

minor DNA population found in patient 3 was eliminated within 3 years. The second DNA population found in patient 2 arose within 6 years.

Eight HBV-DNAs (nos. 2.2A, 3.1A, 3.2, 4.1B, 4.2B, 6.2B, 7, and 9) were identical to the HBV-DNA of patient 1, seven HBV-DNAs (nos. 2.1, 2.2B, 4.1A, 4.2A, 6.1B, 6.2A, and 8) showed one base exchange, two HBV-DNAs (nos. 3.1B and 6.1A) showed two base exchanges, and one DNA (no. 5) showed three base exchanges. The exchanges resulted in alterations of the polymerase, the small surface peptide, and the enhancer I. By contrast, no changes were found in the amino acids of the core and of the X-protein.

Two unique mutations were detected: 57 (C→T) in DNA no. 5 and 234 (C→G) in DNA no. 2.2B. Both mutations resulted in amino acid exchanges in the polymerase at positions 311 (P→S) in the "tether", and 370 (H→Q) in the *N*-terminal region of the reverse transcriptase, respectively. Surprisingly, the mutation at nucleotide position 234 (sample 2.2B) was located in a highly conserved region.

Three nucleotide mutations were located at *cis*-acting elements: 1,130 (A→C), sample no. 8, located in the hepatocyte nuclear factor 3 binding site [Ori and Shaul, 1995] of the enhancer I, 1,163 (A→C) of the DNAs nos. 2.1, 6.1A and 6.1B at the inverse repeat of the transcription factor RFX-1 binding site [David et al., 1995] of the enhancer I. The exchanged 1,163-C found in DNA nos. 2.1 and 6.1 mutated back to A (sample 2.2 and 6.2) within 5 years. The nucleotide exchange 1,163 represented a silent mutation, the exchange at position no. 1,130 resulted in an amino acid substitution (K→N) near the C-terminus of the reverse transcriptase. The silent mutation at position 2,942 (T→C) found in DNA nos. 6.1A and 6.2A was located in a highly conserved region, unique to the HBV ayw subtype.

Reconstruction of Infection Chains

Figure 1 gives the phylogenetic tree obtained when comparing the DNA sequences from the samples of our patients with a series of published HBV-DNAs of the subtype ayw. The nucleotide differences found between our isolates (three at a maximum) were much smaller than those between these and other HBV-DNAs: The 7 most similar HBV sequences published, differed by 18 (Italian isolate "3" [Sterneck et al., 1996], 21 (French isolate NAT [Kremsdorf et al., 1993], 24 (German isolate "2" [Sterneck et al., 1996], 29 (French isolate [Galibert et al., 1979, HBJAYW (EMBL database)], 36 and 40, respectively, (German isolates "1," "3," and "2" [Sterneck et al., 1997] nucleotides from DNA no. 1. Furthermore, four of the nucleotides (465-A, 584-T, 1679-T, and 2848-T) found in our isolates were unique.

These findings confirm the conclusion that the HBV outbreak in our patients originated from one index patient (no. 1) whose HBV triggered the infection chains.

The DNAs nos.2.1, 2.2A, 2.2B, 3.1A, 3.2, 4.1A, 4.1B, 4.2A, 4.2B, 6.1B, 6.2A, 6.2B, 7, 8, and 9 differed by at most one base from the DNA no. 1, the DNAs nos. 3.1B and 6.1A by two, and no. 5 by three bases.

The probable infection chains established by placing

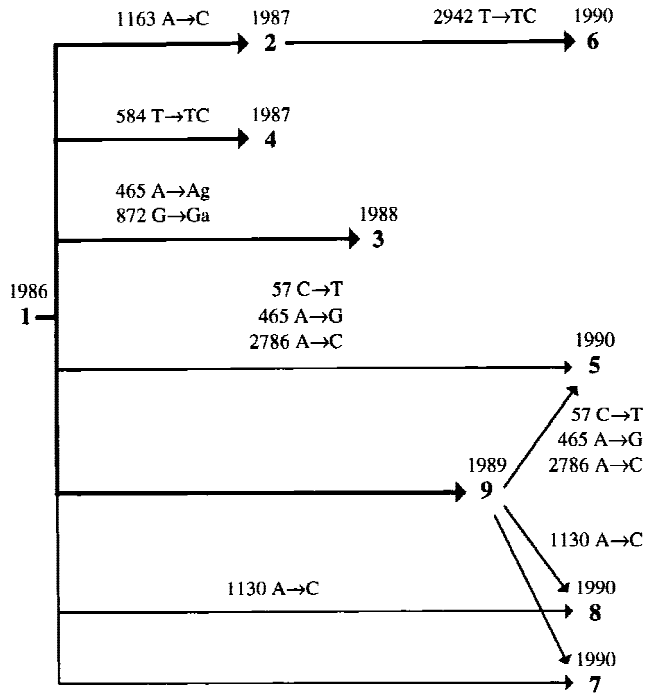

Fig. 2. Reconstruction of hepatitis B virus (HBV) infection chains on the bases of the smallest nucleotide differences between DNAs isolated. Capital letters stand for the main populations, small letters for the minor population, and bold numbers for the patients.

the DNA samples according to the smallest number of base differences and considering the dates of first HBsAg positivity of the patients are given in Figure 2. According to the criteria used, the conclusion was reached that patient no. 1 infected patients 2, 3, 4, and 9 and that patient no. 2 was the source of infection for patient no. 6. Patient no. 5 could have been infected either by patient nos. 1, 3, or 9. However, in contrast to patients 1 and 5, patient no. 3 possessed two different HBV-DNA populations. Assuming that infection chains involving loss of a DNA population are less likely than those without such loss, the conclusion was reached that the probable source of infection of patient no. 5 was either patient no. 1 or 9. Furthermore, patients 7 and 8 were infected by either patient no. 1 or patient no. 9. It cannot be excluded that these probable chains involved further intermediate patients.

Calculation of Evolution Rates

The evolution rates (E) calculated are given in Table IV. A mean value of 0.67×10^{-4} ($\pm 0.85 \times 10^{-4}$ SD) and a high degree of variability were found. The means of the evolution rates recorded for passage of virus between two patients ($E_e = 0.69 \times 10^{-4} \pm 0.93 \times 10^{-4}$ SD; determination nos. 1–18) did not differ significantly (Mann–Whitney test) from those calculated for passage within a given patient ($E_i = 0.63 \times 10^{-4} \pm 0.69 \times 10^{-4}$ SD; determination nos. 19–26).

By GLM-ANOVA, the conclusion was reached that the evolution rate (E) decreased with increasing evolution time (t) ($P = .10$) and did not depend on passing of virus on to one or more patients.

DISCUSSION

In the experiments described above, direct sequencing of PCR amplificates was used instead of sequencing cloned HBV-DNA. Direct sequencing was considered to have the following advantages:

1. Selection of DNA populations is avoided. The main and minor populations (≥10%) can be recognised.
2. Starting the PCR with more than 100 HBV genome equivalents, random mutations caused by the Taq DNA polymerase used (error rate about 10^{-4} [Tindall and Kunkel, 1988; Barnes, 1992]) would yield invisible bands in the sequence ladder obtained by direct sequencing of the amplficates. By contrast, the Taq DNA polymerase would yield different sequence patterns when using cloned PCR products.

The results of sequencing the HBV-DNA of our patients indicated that the hepatitis B outbreak amongst these patients originated from a single patient. This conclusion is based on the finding that all isolates belonged to the same subtype and showed a maximum of three base differences when compared with each other, but differed by at least 18 bases when compared with other HBV-DNA sequences of the same subtype. The infection chains reconstructed on the basis of the similarity of isolates agreed well with those established from epidemiological data [Drescher et al., 1994].

With the exception of samples 4.1A and 4.2A, all the patients' DNA was found to have leucine instead of serine at position 143, located in the middle of the second loop of the epitope "a" of the HBsAg. This rare exchange of a short hydrophilic amino acid by a longer hydrophobic amino acid has also been described for the HBV-DNA of Spanish drug abusers [Wallace et al., 1994] and a child with cirrhosis [Pollicino et al., 1996]. The exchange 143 (L→S) could reflect a reversion to the sequence of the known ayw 3 serotype. Amino acid exchanges in the epitope "a" are known to be associated with altered immunogenicity of the HBV [Carman et al., 1990, 1995; Moriyama et al., 1991; McMahon et al., 1992; Okamoto et al., 1992; Waters et al., 1992; Fortuin et al., 1994; Karthigesu et al., 1994; Yamamoto et al., 1994; Howard and Allison, 1995; Mimms, 1995; Lee et al., 1997]. As a consequence, it could be speculated whether this exchange was essential for the persistence of virus in these patients. However, in patient no. 4, DNA with (4.1B and 4.2B) and without (4.1A and 4.2B) this exchange was found in samples taken at 4-year time intervals, a finding arguing against this interpretation.

The nucleotide exchange 1,163 (A→C) detected in samples 2.1 and 6.1 was located in an inverted repeat (1,152-GTTGCcgGCAAC-1,164) to which the nuclear factor EF-C and the transcription factor RFX-1 bind [David et al., 1995]. Dimeric EF-C and RFX-1 bound to the complete inverted repeat is required for the func-

TABLE IV. HBV DNA Evolution Rates

Determination No.[a]	Numbers of DNAs compared 1st	2nd	Evolution time[c] (years)	Number of Mutations found	Evolution rate E·10^4 (exchanges per base/year)	Mean values (± SD) All determ. E·10^4	Determin. 1–18 E_e·10^4	Determin. 19–26 E_i·10^4
1	1	2.1	1.37	1	2.29			
2	1	2.2A[b]	7.36	0	0			
3	1	2.2B	7.36	1	0.43	0.67	0.69	0.63
4	1	3.1A	2.08	0	0	±0.85	±0.93	±0.69
5	1	3.1B	2.08	2	3.02			
6	1	3.2	5.17	0	0			
7	1	4.1A	2.54	1	1.24			
8	1	4.1B	2.54	0	0			
9	1	4.2A	7.34	1	0.43			
10	1	4.2B	7.34	0	0			
11	1	5	4.55	3	2.07			
12	1	6.1A	4.56	2	1.38			
13	1	6.1B	4.56	1	0.69			
14	1	6.2A	8.89	1	0.35			
15	1	6.2B	8.89	0	0			
16	1	7	4.78	0	0			
17	1	8	5.18	1	0.61			
18	1	9	5.62	0	0			
19	2.1	2.2A	5.99	1	0.52			
20	2.1	2.2B	5.99	2	1.05			
21	3.1A	3.2	3.08	0	0			
22	3.1B	3.2	3.08	2	2.03			
23	4.1A	4.2A	4.79	0	0			
24	4.1B	4.2B	4.79	0	0			
25	6.1A	6.2A	4.32	1	0.73			
26	6.1B	6.2B	4.32	1	0.73			

[a]Determination nos. 1 through 18 compare DNAs obtained from different patients and determination nos. 19 through 26 compare DNAs obtained from different samples fo the same patient.
[b]A and B designate different DNA populations in a sample.
[c]Period of time between drawing blood samples from the patients used for HBV-DNA sequencing.

tional activity of the HBV enhancer I [David et al., 1995]. Therefore, it could be assumed that the activity of enhancer I was reduced in DNAs nos. 2.1 and 6.1 and the back mutation C→A detected in samples 2.2. and 6.2 was required for viral persistence.

Furthermore, it is unlikely that the two nucleotide exchanges located in the middle of the γ-box of the CURS reduced the stimulation of the core promoter, because the γ-box was intact [Yuh et al., 1992]. The base exchange 3,117 (TA) situated near to the 5′-terminus of the region E of the surface promoter, which interacts with transcription factors [Raney et al., 1992] was also found in three other ayw sequences [Kidd-Ljunggren et al., 1995; Sterneck et al., 1996]. The amount of surface antigen (20–200 μg/ml) in the sera studied was in the same range as found in sera of immunocompetent patients with active hepatitis B. Therefore, a marked inhibition of the activity of the surface promoter by the 3,118-A exchange seems unlikely.

In summary, all the exchanges observed did not abolish the ability of HBV to multiply in its host.

The evolution rates of HBV-DNA recorded for our patients agreed well with previously published rates [0.634×10^{-4}, Orito et al., 1989; $0.1–0.5 \times 10^{-4}$, Lin et al., 1991], pertaining to immunocompetent patients. This finding suggests that there is not much immune selection in most of the HBV carriers. Furthermore, because our patients were under severe immunosuppressive medication, this finding indicates that the immunosuppression employed did not influence the evolution rate of HBV.

The average value of the evolution rates determined for the passage of virus within at least two patients ($E_e = 0.69 \times 10^{-4} \pm 0.93 \times 10^{-4}$ SD exchanges per base per year) did not differ significantly from the mean value of the evolution rates (E_i) within a given patient ($E_i = 0.63 \times 10^{-4} \pm 0.69 \times 10^{-4}$ SD). The evolution rates E were found to decrease with increasing evolution time (t) as described by Sterneck et al. [1997]. This finding suggests that shortly after infection, the adaptation of virus to host results in a higher evolution rate, followed by a lower evolution rate during the subsequent persistence of the virus in its host. This interpretation would explain the decline in evolution rates with increasing evolution time and would also account for the high degree of variability of the rates found.

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